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Subgingival biofilm colonization by *Candida albicans* and *Candida dubliniensis* in patients living with HIV from Buenos Aires, Argentina

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ABSTRACT

Oropharyngeal candidiasis (OC) is common among people living with HIV (PLWH). Persistent colonization of oral epithelial surfaces serves as an ecological niche for opportunistic pathogens and is a significant predisposing factor for OC development in PLWH. Mucosal colonization can lead to biofilm formation, directly impacting oral epithelium. ***Aim:*** *To assess Candida albicans and Candida dubliniensis colonization in subgingival biofilms of people living with HIV (PLWH) and undergoing antiretroviral therapy (ART).* ***Materials and Method:*** *A sample of 51 PLWH who were receiving ART was studied, focusing on dental and periodontal parameters. Subgingival biofilm and mucosa samples were collected, and Candida spp. were identified using molecular techniques.* ***Results:*** *Men (average age: 41.11 ± 8.63) predominated. The main cause of HIV was sexual transmission. Fungal-related opportunistic diseases were observed in 18 patients, and LT CD4 counts were evaluated. A total 255 samples were collected, including 204 from gingivo-periodontal sites and 51 from oral mucosa. Candida spp. was detected in 55% of patients, with particular distribution patterns. Positive Candida spp. presence correlated with clinical attachment level and HIV treatments. Microscopic identification revealed the presence of hyphae at the time of microbiological sample collection. Molecular identification confirmed 16 Candida albicans and 36 Candida dubliniensis isolates, challenging their diagnostic importance.* ***Conclusions:*** *The presence of yeast hyphae/pseudohyphae in subgingival biofilms indicates their role in gingivo-periodontal disease dysbiosis. PLWH in this Argentine region face challenges including limited access to healthcare. The study underscores the need for early oral health intervention, emphasizing the diagnostic significance of Candida.*

Key Words: *Candida dubliniensis - Candida albicans - subgingival biofilm - periodontitis - HIV - oral colonization.*

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Colonización de *biofilm* subgingival por *Candida albicans* y *Candida dubliniensis* en pacientes que conviven con el VIH de Buenos Aires, Argentina

RESUMEN

La candidiasis orofaríngea (CO) es común en pacientes que viven con HIV (PVVS). La colonización persistente de las superficies epiteliales orales sirve como reservorio para patógenos oportunistas. Esta colonización es un factor predisponente para el desarrollo de la CO en PVVS. La colonización mucosa puede llevar a la formación de biofilm, impactando directamente en el epitelio oral. ***Objetivo:*** *Evaluar la colonización por Candida albicans y Candida dubliniensis en biofilm subgingival de pacientes que viven con VIH (PVVS) bajo tratamiento antirretroviral.* ***Materiales y Método:*** *Se estudió una cohorte de 51 PVVS bajo TAR, centrándose en parámetros dentales y periodontales. Se recolectaron muestras de biofilm subgingival y mucosa, identificando Candida spp. mediante técnicas moleculares.* ***Resultados:*** *Predominaron hombres con edad promedio de 41,11 ± 8,63, siendo la transmisión sexual la principal vía. Se observaron enfermedades oportunistas relacionadas con hongos en 18 pacientes, evaluándose los recuentos de CD4 a largo plazo. En total, se recopilaron 255 muestras, incluyendo 204 de sitios gingivo-periodontales y 51 de mucosa oral. Se detectó Candida spp. en el 55% de los pacientes, con patrones de distribución particular. La presencia positiva de Candida spp. se correlacionó con nivel de inserción clínica y TAR. La identificación microscópica reveló la presencia de hifas al momento de la toma de muestras microbiológicas. La identificación molecular confirmó 16 aislamientos de Candida albicans y 36 de Candida dubliniensis, desafiando su importancia diagnóstica.* ***Conclusiones:*** *La presencia de hifas/pseudohifas de levaduras en biofilm subgingival indica su papel en la disbiosis de enfermedades gingivo-periodontales. Los PVVS en esta región argentina enfrentan desafíos debido al acceso médico limitado. El estudio destaca la necesidad de intervenciones tempranas en la salud oral, enfatizando la importancia diagnóstica de Candida spp.*

Palabras Clave: *Candida dubliniensis - Candida albicans - biofilm subgingival - periodontitis - VIH - colonización oral.*

INTRODUCTION

Oropharyngeal candidiasis (OC) is prevalent among people living with HIV (PLWH). Most of these infections arise from *Candida albicans (C. albicans)*, although a global rise in cases attributed to *non-albicans Candida* species in recent years has been reported. OC is an immunosuppression indicator, notably prevalent in patients with a Lymphocyte T CD4 count below 200 cells/ml. OC manifests in approximately 95% of people during stage 1 HIV. With the introduction of antiretroviral therapy (ART), there has been a significant reduction in the incidence of HIV-related oral pathologies, including OC, leading to a decline in systemic disease cases[2]. Nevertheless, the persistent colonization of epithelial surfaces in the oral cavity, which serve as the natural ecological niche for this opportunistic pathogen, remains a robust predisposing factor for the development of OC in PLWH, even if they are under stable treatment with a combination of ART. It is estimated that 90% of infected patients will experience OC at some point during the evolution of HIV infection[1,3,4]. Colonization of mucosal surfaces can give rise to the formation of biofilms, exerting direct impact on the oral epithelium. Biofilm formation induces alterations in cell structure and function, affecting innate immunity and creating an environment conducive to colonization and infection by commensal and pathogenic microorganisms[5]. The virulence of these microorganisms depends on the gene expression associated with their morphotypes. It is important to note that although such colonization does not invariably lead to candidiasis, it is a prerequisite for its onset[4,6-9]. This situation persists despite ART and may be correlated with the residual presence of HIV in mucosal macrophages and dendritic cells[10,11].

There is a broad range of risk factors that predispose individuals to colonization, including smoking, diabetes mellitus, use of oral prostheses, advanced age, use of antibiotics, reduced salivary flow, dietary habits, nutritional status, inadequate oral hygiene, and immunosuppression such as HIV, among others[6,8,12].

Because *Candida dubliniensis (C. dubliniensis)* has become increasingly prevalent in various countries, studies have been conducted to compare its virulence to that of *C. albicans*. *C. dubliniensis* was initially characterized in 1995 by Sullivan et al.[13], and isolated mainly from the oral cavities of PLWH. *C. dubliniensis* was first identified in the subgingival biofilm of periodontal lesions in PLWH in 2001[13,14]. It is known that *C. dubliniensis* can be isolated from individuals with different local or systemic pathologies, and may even colonize the subgingival biofilm of healthy people. In the last five years, *C. dubliniensis* has been associated with the emergence of more recent complications such as meningitis, endocarditis, and recurrent oral and respiratory diseases, particularly in immunocompromised patients[15-17].

The aim of this study was to assess *C. albicans* and *C. dubliniensis* colonization in subgingival biofilm of patients living with HIV (PLWH) undergoing antiretroviral therapy (ART).

MATERIALS AND METHOD

This was an analytical, descriptive, prospective, cross-sectional study. A non-probabilistic sequential sample of PLWH seeking care through spontaneous demand was established at three healthcare facilities in Buenos Aires City (Clínica para la atención de pacientes de alto riesgo (CLAPAR I) University of Buenos Aires, Faculty of Dentistry; the Hospital General de Agudos Dr. Juan A. Fernández, and Hospital de Enfermedades Infecciosas Francisco Javier Muñiz"). The study was approved by the Ethics Committee of University of Buenos Aires, Faculty of Dentistry, with protocol number 002/2019-CETICA-FOUBA). Procedures were carried out after patients were informed about the protocol and voluntarily signed informed consent,

The study included PLWH aged 18 to 60 years with detectable HIV viral load determined by qPCR (Real-Time PCR) using the HIV-1 viral load assay (version 3.0 Abbott), the most recent LT CD4 count, who had been on highly active antiretroviral therapy (HAART) for at least six months before study enrollment. Exclusion criteria were systemic illnesses unrelated to HIV infection, treatment with antibiotics, antifungals, or oral antiseptics within the three months preceding microbiological tests, oropharyngeal or systemic candidiasis lesions, and receiving dental care from other service providers.

Composition of the study population

The study population comprised 59 individuals who underwent a medical history assessment, including the recording of socio-demographic data, prior

opportunistic diseases, and hospitalization history. The patients were evaluated by a calibrated dental professional (Kappa ≥0.80). Dental status and periodontal clinical parameters were documented using Marquis-type periodontal probes at six sites per tooth, considering probing depth (PD) in millimeters, clinical attachment level (CAL) in millimeters, and bleeding on probing (BOP) classified as positive or negative. Radiographic periodontal parameters were also examined[18,19].

Sample collection

Samples were collected according to the following protocol:

1. Mouth rinse with sterile distilled water for one minute.
2. Oral cavity inspection with proper illumination and a dental mirror.
3. Swabbing of all mucosal surfaces using a sterile Dacron swab.
4. Partial isolation of the targeted periodontal site with a sterile cotton roll and suction.
5. Identification of 4 periodontal sites based on clinical-radiographic criteria.
6. Removal of supragingival biofilm (if present) with a Gracey curette (Hu-Friedy) corresponding to the tooth number.
7. Placing sterile paper points (Meta, Biomed) #30-35 into the gingival sulcus or periodontal pocket.
8. Recording periodontal conditions.
9. Collection of subgingival biofilm from the epithelial pocket for smearing on a sterile microscope slide.

Each sample was placed in a tube containing Reduced Transport Fluid (RTF) and transported refrigerated to the laboratory under biosecurity conditions[20] (Fig. 1).

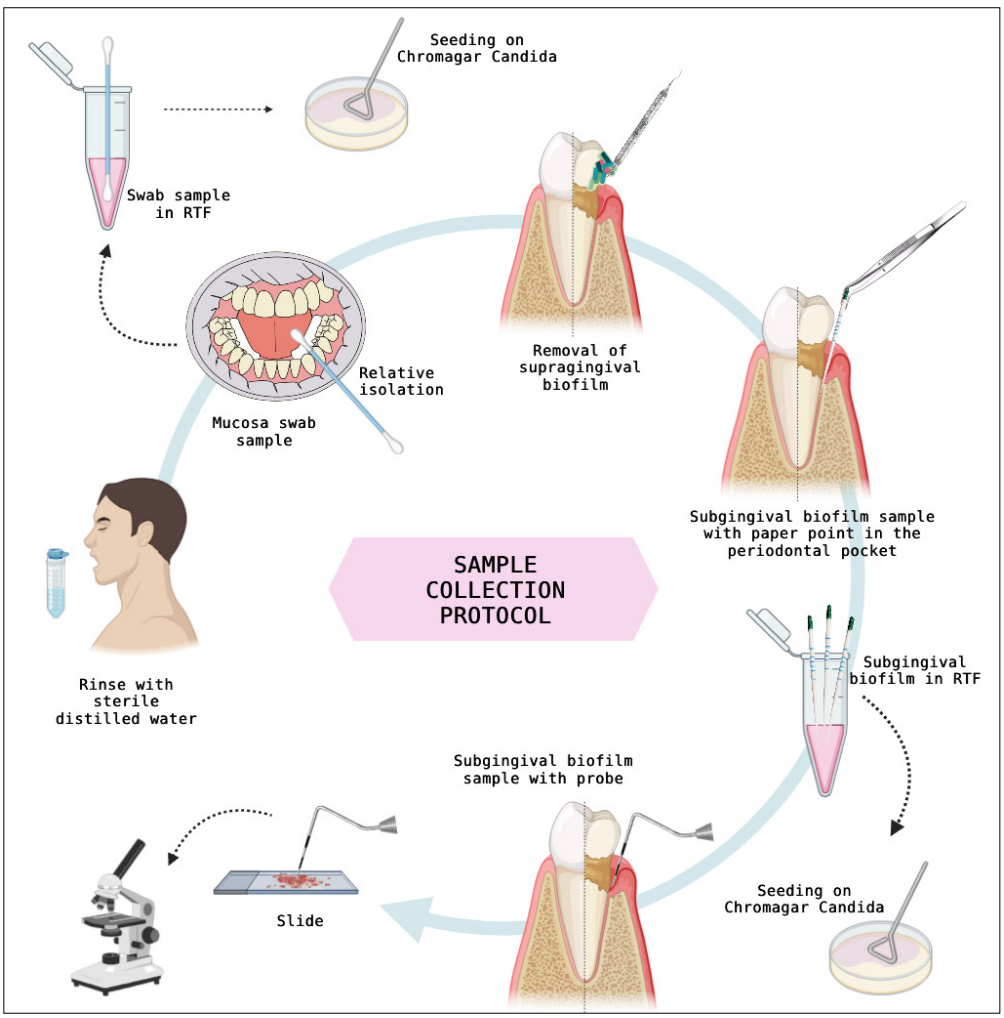

Fig. 1: Sample collection protocol. Created with BioRender.com

Molecular identification of *Candida* species

Each sample was vortexed for one minute. Subsequently, 100 µl of the sample was plated on Chromagar *Candida*® (CHROMagar, France) and incubated at 37 ºC for 48 hours under aerobic conditions, facilitating the presumptive identification of *Candida* spp. The colonies obtained were sub-cultured on Sabouraud Dextrose Agar (SDA) at 37 ºC for 24 hours under aerobic conditions to conduct phenotypic presumptive identification tests[21].

Genomic DNA extraction

Fresh cultures were incubated for 24 hours on Yeast Peptone Dextrose agar (YPD) to extract genomic DNA (gDNA) from a colony of each isolate. Two extraction methods were employed: the commercial Yeast Genomic DNA Kit (Zymo Research, USA) following the manufacturer's instructions, and the commercial Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) with some modifications. The gDNA was quantified in triplicate using the Cytation 3 Cell Imaging multi-mode reader (BioTek, USA)[21].

qPCR Multiplex of ITS regions

Two species-specific primers derived from the internal transcribed spacer (ITS), ITS-1, 5.8S rRNA, and ITS-2 regions of the ribosomal DNA (rDNA) were used with some modifications[7]. The amplification process was assessed using CFX Maestro Software (Bio-Rad Life Science, USA) through melting curve analysis, with a temperature of 86 ºC (±0.5) for C. *albicans* and 82 ºC (±0.5) for C. *dubliniensis*[7].

Multiplex PCR of HWP1 gene

To optimize detection strategies, the HWP1 gene was amplified following the amplification protocol described by Dubois et al. 2023[21], with some modifications, in an Aeris-BG096 thermocycler (Esco Scientific, Singapore). The final amplification products were separated by electrophoresis using 1.3% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer with the addition of 1.5 µl of GelGreen® (Biotium, USA), and visualized using the GelDoc XR+ Gel Documentation System (Bio-Rad Life Science, USA).

The presence of different alleles for the HWP1 gene can be determined[22]. *C. albicans* ATCC 10231 and *C. dubliniensis* CD36 were used as positive reference controls, and *Candida parapsilosis* ATCC 22019 as a negative control[21].

A descriptive statistical analysis was conducted, determining relative frequency expressed in percentages, means/medians, standard deviations and confidence intervals for quantitative variables. Student's t-test was applied to compare quantitative variables. For categorical variables, the chi-square statistic (χ^2) was used. Differences were considered significant when $p<0.05$. The tests were conducted using the software SPSS (v.29.0) and Google Sheets implemented in Google Drive 2023.

RESULTS

In the present study, an initial sample of 59 PLWH undergoing HAART was evaluated. Eight patients were excluded due to unclear medical history regarding the mode of transmission or lack of data. Consequently, the final study population was reduced to 51 patients, representing 86.44% of the initially considered total.

The final study population consisted of 70.6 % males, and average age was 41 ± 8. According to the collected medical history data, the primary mode of HIV transmission was sexual. Concerning the history of HIV-related hospitalizations, most patients had not been hospitalized before. Regarding past opportunistic diseases, 18 patients had had fungal-related opportunistic diseases, of whom 6 had records of antifungal treatment in their medical history, with 1 treated solely with fluconazole, 1 with nystatin and fluconazole, and 1 with fluconazole and voriconazole. The antifungal type was unknown for the remaining cases.

LT CD4 count was evaluated with two cutoff values, 200 cells/ml and 400 cells/ml or higher. Notably, one patient's value was unknown, so the LT CD4 data correspond to the analysis of 50 patients. Details are presented in Table 1.

In relation to HAART treatment, only 3 patients were exclusively taking non-nucleoside analogs. The rest were taking a combination of two or three ART agents. Notably, no patient was exclusively on nucleoside analogs or protease inhibitors (Fig. 2) (Table 2).

Characteristics and composition of the selected sample

A total of 255 samples were collected from the 51 included patients, of which 204 corresponded to

Table 1. Medical and socio-demographic data.

Patients (n=51)		Frequency	%
Gender	Female	10	19.6%
	Male	36	70.6%
	Non-binary	5	9.8%
Nationality	Argentine	46	90.2%
	Foreign	5	9.8%
Residence	CABA	41	80.4%
	AMBA	9	17.6%
	Outside AMBA	1	2%
Employment	Employees	18	35.3%
	Self-employed	17	33.3%
	Students	3	5.9%
	Retirees	1	2%
	Unemployed	10	19.6%
	No response	2	3.9%
Health insurance	Yes	12	23.5%
	No	39	76.5%
HIV transmission	Sexual	46	90.2%
	PDUP	4	7.8%
	Vertical	1	2%
HIV hospitalization history	Yes	17	33.3%
	No	34	66.7%
Opportunistic diseases	Yes	18	35.3%
	No	33	64.7%
Co-infection TB	Yes	1	2%
	No	50	98%
Co-infection HBV	Yes	5	9.8%
	No	46	90.2%
Co-infection HBC	Yes	3	5.9%
	No	48	94.1%
Viral load	≤50	43	84.3%
	> 50	8	15.7%
Patients (n=50)		**Frequency**	**%**
LT CD4	≤200	6	12%
	>200	44	88%
LT CD4	≤400	23	46%
	>400	27	54%

CABA: Buenos Aires Autonomous City, AMBA: Buenos Aires Metropolitan area, PDUP: Parenteral Drug User, TB: Tuberculosis, HBV: Hepatitis B, HCV: Hepatitis C.

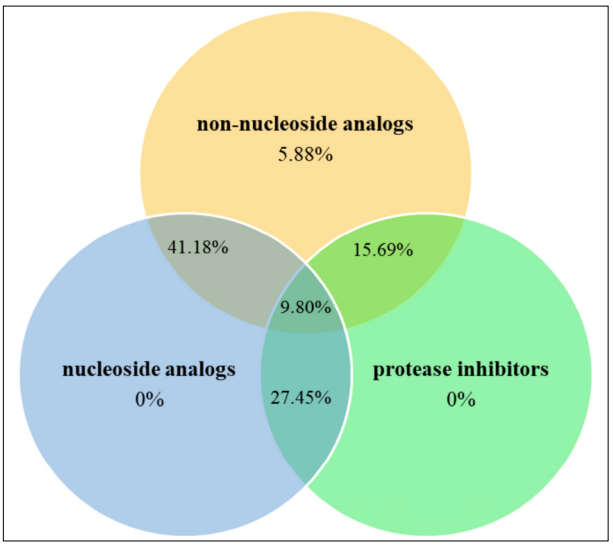

Fig. 2: Percentage and combination of HAART treatments in patients.

Table 2. Number of patients (N) receiving the specified antiretroviral (ART) in the HAART combination.

HAART	Type of ART	N
Nucleoside analogs	ZIDOVUDINE (AZT)	12
	LAMIVUDINE (3TC)	34
	ABACAVIR	14
	DIDANOSINE (ddi)	1
Non-nucleoside analogs	NEVIRAPINE	6
	EFAVIRENZ	17
	EMTRICITABINE (FTC)	9
	TENOFOVIR	18
Protease inhibitors	SAQUINAVIR	1
	LOPINAVIR	10
	RITONAVIR	25
	ATAZANAVIR	7
	FOSAMPRENAVIR	3
	DARUNAVIR	3
	MARAVIROC	1

gingivo-periodontal sites and 51 to oral mucosa samples.

According to the classification of periodontal and peri-implant diseases and conditions, 9 patients (17.65%) were in a state of eubiosis, 5 (9.80%) were diagnosed with gingivitis, and 37 (72.55%) were diagnosed with periodontitis. Among those with periodontitis, 10 (27.03%) were classified as stage I, grade A, 17 (45.95%) as stage II, grade B, and 10 (27.02%) as stage III, grade B.

Regarding the gingivo-periodontal parameters used for subgingival biofilm sampling (n=204), BOP was positive at 121 sites (47.5%), PD > 3 mm at 96 sites

(37.6%), PD ≤ 3 mm at 159 sites (62.4%), CAL > 1 mm at 139 sites (54.5%), and no CAL loss was recorded at 116 sites (45.5%).

Considering *Candida* spp. as the unit of analysis in the 255 collected samples, 28 (55%) patients yielded positive results for the presence of *Candida* spp., representing a total of 82 positive samples collected. Among these, 65/204 (32.4%) were from gingival and periodontal sites, while 17/51 (33.3%) were from mucosal samples.

Different distribution patterns of the samples were observed: yeast was exclusively isolated from the mucosa in one patient (3.6%). In 11 patients (39.3%), it was isolated solely from gingivo-periodontal sites. In 16 (57.1%), presence was detected in both gingivo-periodontal sites and mucosa. In 8 patients (28.6%), *Candida* spp. was isolated from all collected samples, including the 4 gingivo-periodontal sites and the mucosa sample.

Only the presence of *Candida* spp. in gingivo-periodontal sites with negative BOP was statistically significant ($p<0.01$). The association with other parameters is shown in Table 3.

At gingival and periodontal sites, the presence of *Candida* spp. was positive at 34 sites (40.5%) that did not exhibit gingival bleeding on probing (BOP), and this result was statistically significant ($p<0.05$). Regarding probing depth (PD), *Candida* spp. was positive at 26 sites (27.1%) with PD >3 mm and 38 sites (35.2%) with PD ≤ 3 mm. The presence of *Candida* spp. was higher at sites without probing depth. Concerning clinical attachment level (CAL), *Candida* spp. was positive at 39/139 sites (28.1%) where CAL was detected in 25/65 sites (38.5%) without CAL. The finding of *Candida* spp. was higher at sites with CAL >1 mm (Table 3).

Analysis of HIV-related variables, focusing on the HAART treatment in the 28 patients positive for *Candida* spp., showed that 3 (10.71%) were being treated with a combination of nucleoside analogs, non-nucleoside analogs and protease inhibitors, 8 (28.57%) with non-nucleoside and nucleoside analogs, 7 (25.0%) with protease inhibitors and non-nucleoside analogs, and 10 (35.72%) with protease inhibitors and nucleoside analogs.

Regarding viral load, *Candida* spp. was detected in 22 patients (78.58%) with viral load ≤50 copies/ml and in 6 patients (21.42%) with viral load > 50 copies/ml.

Considering the LT CD4 count and excluding the patient with unknown CD4 value, *Candida* spp. was detected in 13 patients (48.15%) with ≤400 cells/ml and 14 patients (51.85%) with >400 cells/ml.

Concerning the CD4 count cutoff at 200 cells/ml, *Candida* spp. was found in 24 patients (88.89%) with values ≥200 cells/ml, and 3 patients with values <200 cells/ml. Among the latter three patients, 2 were in eubiosis, and one had periodontitis. Three other patients in similar conditions were negative for *Candida* spp. On the other hand, *Candida* spp. was detected in 6 patients with viral load >50 copies/ml. Only one of them had CD4 <200 cells/ml, viral load >50 copies/ml and periodontitis. Six additional patients with similar results tested negative for *Candida* gender.

The relationship of viral load and CD4 count with opportunistic infections and co-infections

Table 3. Isolations of *Candida* spp. according to gingivo-periodontal parameters.

Gingivo-periodontal sites (n=204)			***Candida* spp.**			
	Positive		Negative			
	Frequency	%	Frequency	%	p value	
BOP	Positive	30	25.0%	90	75.0%	0.019
	Negative	34	**40.5% ***	50	59.5%	
PD	> 3 mm	26	27.1%	70	72.9%	
	≤ 3 mm	38	35.2%	70	64.8%	
CAL	> 1 mm	39	28.1%	100	71.9%	
	≤ 1 mm	25	38.5%	40	61.5%	

BOP: bleeding on probing, PD: probing depth and CAL clinical attachment level. * **p** <0.05.

parameters in PLWH is shown in Tables 4 and 5.

Table 4. Relationship among CD4 count cutoff 200 cells/ml with opportunistic infections, co-infections and Candida spp.

Patients (n= 50)		Lymphocyte T CD4	
	<200 cells/ml	≥ 200 cells/ml	
	Frequency		
Candida spp	Positive	50%	54.5%
	Negative	50%	45.5%
Fungal diseases	Yes	100%	31.8%
	No	0%	68.2%
Co-infection TB	Yes	0%	2.3%
	No	100.%	97.7%
Co-infection HBV	Yes	16.7%	11.4%
	No	83.3%	88.6%
Co-infection HBC	Yes	16.7%	4.5%
	No	83.3%	95.5%
Viral load	≤ 50	66.7%	86.4%
	> 50	33.3%	13.6%

TB: Tuberculosis, HBV: Hepatitis B, HCV: Hepatitis C. The values correspond to 50 patients due to an unknown count in one of them.

Table 5. Relationship among viral load count cutoff 50 copies/ml with opportunistic infections, co-infections and Candida spp.

Patients (n= 50)		Viral load	
		< 50 copies/ml	≥ 50 copies/ml
		Frequency	
Candida spp	Positive	57.9%	50%
	Negative	42.1%	50%
Fungal diseases	Yes	33.3%	50%
	No	66.7%	50%
Co-infection TB	Yes	2%	50%
	No	98%	50%
Co-infection HBV	Yes	9.3%	14.3%
	No	90.7%	85.7%
Co-infection HBC	Yes	4.8%	12.5%
	No	95.2%	87.5%
LT CD4	< 200	47.6%	25%
	≥ 200	52.4%	75%

TB: Tuberculosis, HBV: Hepatitis B, HCV: Hepatitis C. The values correspond to 50 patients due to an unknown count in one of them.

The collection of subgingival biofilm facilitated the acquisition of samples for a smear, especially at sites with active periodontal disease. The presence of yeast and/or hyphae was observed, along with the periodontopathogenic bacterial microbiota and the inflammatory response. These findings are consistent with the presence of *Candida* spp. infection and other microorganisms associated with periodontal disease. The presence of yeast and hyphae alongside the bacterial microbiota suggests a potential interaction between these microorganisms and their potential contribution to the pathogenesis of gingival and periodontal diseases (Fig. 3).

The molecular identification of the yeasts was previously published by our laboratory[21]. It was determined that 16 isolates corresponded to *C. albicans*, and 36 to *C. dubliniensis*. Four atypical colonies were identified as *C. dubliniensis*. Ten strains tentatively identified could not be recovered. The remaining yeast species tentatively identified, which tested negative in both PCR methods employed, did not belong to either species, and their definitive identification was not conducted. With regard to patients with more than one *Candida*

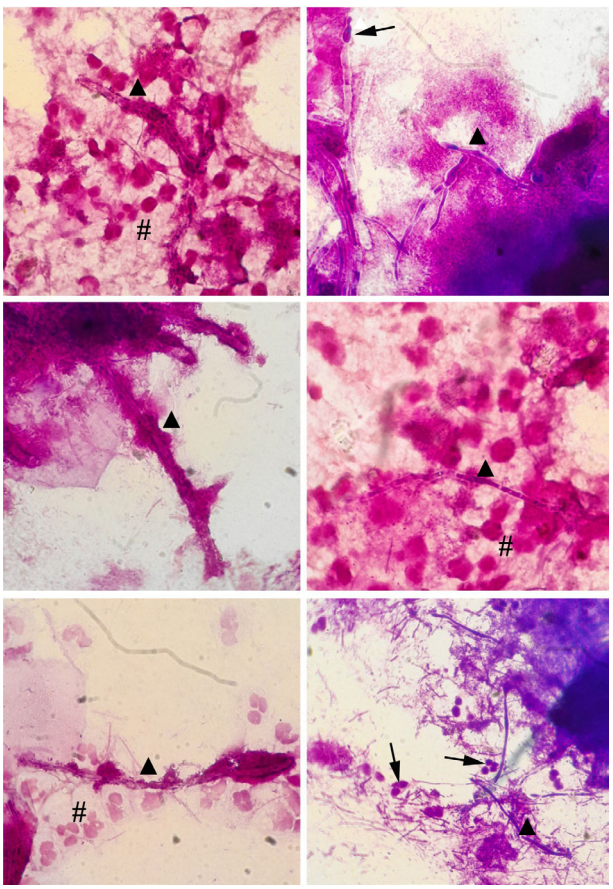

Fig. 3: Micromorphology of subgingival biofilm: Pseudomycelia/hyphae (▲), yeast (↑) and host's inflammatory response cells (#). Gram stain at 1000X

species at the same periodontal or mucosal site, the following findings were observed: In three patients, *C. albicans* and *C. dubliniensis* were found simultaneously at one periodontal site. In one of these patients, three different morphotypes of *C. dubliniensis* with atypical macroscopic and microscopic aspects were present. Two patients exhibited presence of *C. dubliniensis* along with a species compatible with *Nakaseomyces glabrata* (*N. glabrata)* at the same periodontal site. In another patient, *C. albicans* was detected along with a species compatible with *N.glabrata* at one periodontal site. At a mucosal site of another patient, *C dubliniensis* coexisted with a species compatible with *Pichia kudriavzevii* (formerly *Candida krusei*) (Table 6).

Table 6. Candida isolates according to the periodontal status and isolation sites. Only species with molecular confirmation are shown.

Patient	Sample	Diagnosis	*Candida*
01-0-C	periodontal	Periodontitis, S II, G B	*C.albicans*
01-0-C	periodontal	Periodontitis, S II, G B	*C.albicans*
01-0-C	periodontal	Periodontitis, S II, G B	*C.albicans*
01-0-C	periodontal	Periodontitis, S II, G B	*C.albicans*
03-0-C	periodontal	Periodontitis, S III, G B	*C.dubliniensis*
03-0-C	periodontal	Periodontitis, S III, G B	*C.albicans*
03-0-C	periodontal	Periodontitis, S III, G B	*C.albicans*
06-0-C	periodontal	Periodontitis, S I, G A	*C.albicans - C.dubliniensis* *
06-0-C	periodontal	Periodontitis, S I, G A	*C.dubliniensis*
07-0-C	periodontal	Eubiosis	*C.dubliniensis* *
08-0-C	periodontal	Eubiosis	*C.albicans - Candida* sp.
10-0-C	periodontal	Periodontitis, S II, G B	*C.albicans*
10-0-C	periodontal	Periodontitis, S II, G B	*C.albicans*
10-0-C	mucosa	Periodontitis, S II, G B	*C.dubliniensis- Candida* sp.
11-0-C	periodontal	Periodontitis, S III, G B	*C.albicans*
20-0-C	periodontal	Gingivitis	*C.albicans - C.dubliniensis*
20-0-C	periodontal	Gingivitis	*C.dubliniensis*
20-0-C	periodontal	Gingivitis	*C.dubliniensis - Candida* sp.
22-0-C	periodontal	Periodontitis, S I, G A	*C.dubliniensis - Candida* sp.
22-0-C	periodontal	Periodontitis, S I, G A	*C.dubliniensis*
22-0-C	periodontal	Periodontitis, S I, G A	*C.dubliniensis*
23-0-C	periodontal	Periodontitis, S II, G B	*C.dubliniensis*
23-0-C	mucosa	Periodontitis, S II, G B	*C.dubliniensis*
24-0-C	periodontal	Eubiosis	*C.albicans - C.dubliniensis* *
25-0-C	periodontal	Eubiosis	*C.dubliniensis*
25-0-C	mucosa	Eubiosis	*C.dubliniensis* *
25-0-C	periodontal	Eubiosis	*C.dubliniensis*
25-0-C	periodontal	Eubiosis	*C.dubliniensis*
25-0-C	periodontal	Eubiosis	*C.dubliniensis*
27-0-C	periodontal	Eubiosis	*C.albicans*
28-0-C	mucosa	Eubiosis	*C.albicans*
29-0-C	periodontal	Periodontitis, S I, G A	*C.dubliniensis*
33-0-C	periodontal	Periodontitis, S II, G B	*C.albicans*
39-0-C	periodontal	Eubiosis	*C.dubliniensis*
39-0-C	periodontal	Eubiosis	*C.dubliniensis*
39-0-C	periodontal	Eubiosis	*C.dubliniensis*
39-0-C	periodontal	Eubiosis	*C.dubliniensis*
39-0-C	mucosa	Eubiosis	*C.dubliniensis*
50-0-C	periodontal	Periodontitis, S II, G B	*C.dubliniensis*
50-0-C	periodontal	Periodontitis, S II, G B	*C.dubliniensis*
55-0-C	periodontal	Gingivitis	*C.dubliniensis*
57-0-C	mucosa	Periodontitis, S I, G A	*C.dubliniensis*
58-0-C	periodontal	Periodontitis, S II, G B	*C.dubliniensis*

E: stage, G: grade, *C.albicans: Candida albicans, C.dubliniensis: Candida dubliniensis.*
*: sites with presence of *C.dubliniensis* typical and atypical morphology.

DISCUSSION

This study characterized and identified *C. albicans* and *C. dubliniensis* isolated from subgingival biofilm and oral mucosa samples of PLWH, whose compromised immune systems make them notably susceptible to opportunistic infections. The selection of the sample was crucial to explore the potential involvement of these species in the etiology of gingival and periodontal diseases within a specific clinical context.

Males constituted the majority in the population sample. Many of the patients examined encountered obstacles in obtaining healthcare. More than half of them lacked health coverage, and approximately one fifth was unemployed. These variables increase patient susceptibility concerning both health and socioeconomic status in the context of HIV.

According to the latest data from the National Health Surveillance System in Argentina, 164.947 people with HIV have been recorded, of whom 65% receive care in the public health system. The report showed that the infection rate is higher in males than females, and a yearly decrease in mortality rates is observed[22,23]. Only one third of the participants in our study had documented antifungal treatment in their medical history, suggesting a deficiency in regular monitoring and follow-up for these patients within the national health system. Some of the protocolized patients presented co-infections with TBC, HCV and HBV. Unfortunately, we lacked data on the follow-up of these co-infections and HIV controls due to the absence of records in the general medical history of the patients.

A decrease in LT CD4 count below 200 cells/ml and a viral load exceeding 50 copies/ml may indicate disease progression to AIDS[24]. Only a minimal proportion of the patients met these conditions.

Over the past decade, there has been significant progress in comprehending this disease. A direct outcome of this advancement is the steadily increasing life expectancy for PLWH, especially in developing nations. The introduction of HAART has profoundly influenced HIV infection, markedly diminishing the morbidity and mortality linked with AIDS, thereby transforming it into a manageable chronic condition[25,26]. It is recommended to start HAART therapy promptly, irrespective of LT CD4 levels and viral load[27]. All patients included in this study were undergoing antiretroviral treatment. Individuals who contracted HIV after 2010 initiated treatment at the time of diagnosis. However, a significant majority of patients diagnosed before 2010 commenced treatment only when their CD4 cell count decreased and/or viral load increased.

The treatment with ART does not completely restore immunity, which gives rise to various complications associated with inflammation and immunodeficiency[26]. Furthermore, the extended life expectancy of these patients has exposed them to a spectrum of chronic diseases associated with aging, which may manifest prematurely and potentially be exacerbated by HIV and HAART[26,28,29].

ART treatment and immunity modulation suggested that periodontitis is more closely linked to the pathogenesis of gingival and periodontal diseases themselves and the increase in life expectancy than with HIV infection. Therefore, considering periodontitis to be an oral disease associated with HIV remains a topic of controversy and debate[30,31].

In this study, most patients had periodontitis, while a smaller group had gingivitis or eubiosis. Recent studies suggest that gingival tissues might serve as a reservoir for HIV due to the substantial presence of inflammatory lymphocytes in periodontal disease. Observations have indicated that the virus found in the gum does not come from peripheral blood, implying that it might be linked to a reactivation of viral replication within the oral mucosa in the context of chronic periodontitis[29,30,32]. One hypothesis suggests that HIV infection may trigger destructive processes in the oral mucosa, similar to those observed in the gastrointestinal tract, potentially aiding the translocation of microorganisms, and may be influenced by the presence of pro-inflammatory taxa in an environment marked by chronic inflammation associated with early-stage HIV. This could result in changes in the gastrointestinal microbiota, ultimately contributing to an increased likelihood of such translocation[28-30].

Concerning HAART, there is evidence suggesting that non-nucleoside analogs might exacerbate or hasten the onset of chronic diseases, particularly those associated with aging. They are also correlated with decreased bone mass and vitamin D deficiency, directly influencing periodontitis. ART may contribute to oxidative stress in the progression of periodontitis. Furthermore, they can heighten endothelial permeability by suppressing essential binding proteins necessary for the proper functionality of the epithelial barrier. The augmentation of capillary permeability is

particularly linked to inflammatory diseases[26,28-30,33]. Protease inhibitors in combination with nucleoside analogs were associated with a lower diversity of oral microbiota compared to both nucleoside analogs and non-nucleoside analogs[28]. In this research, most patients were under treatment with a combination of nucleoside analogs and non-nucleoside analogs.

According to more recent studies, *C. albicans* is an underlying factor capable of triggering microbial dysbiosis and leaky gut syndrome, emphasizing its significance even in the era of ART. Colonization by commensal strains and the emergence of *non-albicans* species can contribute to oral diseases, disseminated infections, and an increase in antifungal resistance, thereby constituting a public health concern, particularly in resource-limited settings[1,11,34,35].

One study suggesting that colonization at periodontal sites may originate from saliva[36] identified only a few patients in whom *Candida* spp. were isolated from both periodontal sites and mucosa.

Annavajhala et al. observed significantly lower fungal diversity in the subgingival biofilm of teeth with severe attachment loss (≥ 4 mm). Additionally, various taxa of bacteria and fungi were enriched in patients with severe periodontitis[28]. In the current study, the isolation frequency of *Candida* species was lower from periodontal sites with clinical attachment loss.

The heightened viral replication and significant depletion of LT CD4 in the oral mucosa lead to a reduction in the production of interleukin 17 and 22, resulting in systemic immune activation and a potential exacerbation of periodontitis[29,30]. On the other hand, Li et al. have suggested that asymptomatic carriage of oral yeasts was associated with a low LT CD4 cell count (≤200 cells/ml), and this value could serve as a predictive factor for yeast colonization[9]. However, in the current study, it was not possible to identify a pattern that would establish a relationship between the isolation of *Candida* and a decrease in LT CD4 cell count or an increase in HIV viral load in the context of gingivo-periodontal diseases.

It was originally believed that *C. dubliniensis* was not associated with systemic diseases, which suggested that the oral cavity could be its natural ecological niche[37]. Nevertheless, this species has garnered escalating attention in clinical research in recent years. Despite its low incidence in cases of candidiasis, its close phylogenetic relationship to *C. albicans* suggests that *C. dubliniensis* may have long been underestimated in candidiasis diagnosis. While most research has focused on the pathogenicity of *C. albicans,* it is crucial to acknowledge the medical significance of other members of the Candida genus, particularly in regions where their prevalence is unknown. Current medical practices, including the preventive or indiscriminate use of antifungals, seem to be contributing to the rise in the prevalence of alternative species[21,38-41].

While *C. albicans* is the most frequently isolated yeast from the oral cavity, the current research revealed a higher proportion of *C. dubliniensis* isolations, with a very low incidence of co-isolation with these species. In a prior investigation conducted by our laboratory, assessing PLWH with and without HAART, a greater number of *C. dubliniensis* isolations were observed in patients undergoing treatment, while the proportion of *C. albicans* was higher in those not receiving ART[7]. This suggests that ART may act as a regulator of the balance between colonization and infection by *C. dubliniensis*.

The presence of *Candida* spp. filaments in the subgingival microbiota were confirmed in some smears performed on the soft wall of the periodontal pocket. Molecular confirmation of these samples enabled the identification of the strains as *C. albicans* and *C. dubliniensis*. Confirmation of the involvement of these species in the infectious stage of periodontitis emphasizes the importance of conducting smears in microbiological studies to help identify the predominant microbiota composition, understand microbial interactions, and discern their impact on the colonization, infection and persistence of microorganisms in specific environments.

Evidence suggests that the subgingival biofilm microbiota differs taxonomically from microbiota isolated from other niches in the oral cavity[7,42]. To assess the alteration in microbiota composition associated with periodontitis, the subgingival biofilm sample is considered the most representative. DNA sequencing techniques developed in recent decades have revealed that various niches in the oral cavity host significantly different microbial communities with distinct compositions[43].

CONCLUSIONS

C. dubliniensis stood out as the most frequently isolated species in the examined population. This

is a significant discovery because the diagnostic importance of this species has been underestimated, with colonization primarily ascribed to *C. albicans*. The presence of yeast hyphae/pseudohyphae in the subgingival microbiota substantiates the involvement of these species in the dysbiosis of gingival and periodontal diseases.

The HIV population in this region of Argentina is clearly vulnerable, with socioeconomic difficulties and limited access to healthcare. The findings of this study underscore the need to address the oral health of these patients at an early stage, aiming to diagnose gingivo-periodontal diseases and understand their implications in fungal infections.

CONFLICT INTERESTS

The authors declare no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

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